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Cell Biology of Mechano-Adaptive Bone Remodeling

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Bone tissue can adapt to changing mechanical demands, but how bone cells may sense and transduce mechanical signals derived from bone loading is still not clarified. The osteocytes are believed to play a role as the "professional" mechanosensory cells of bone, and the lacuno-canalicular network as the structure that mediates mechanosensing. The regulatory process of mechanical adaptation produces flow of interstitial fluid in the bone lacunar-canalicular network along the surface of osteocytes, which is likely the physiological signal for bone cell adaptive responses *in vivo*. The maintenance of a mechanically efficient architecture likely depends on a balance between the intensity and spatial distribution of the mechanical stimulus and the responsiveness of the bone cells. The alignment of secondary osteons along the dominant loading direction suggests that bone remodeling is guided by mechanical strain throughout life at each remodeling cycle. We propose that alignment during remodeling occurs as a result of different canalicular flow patterns around cutting cone and reversal zone during loading.
The response of cultured bone cells to fluid flow includes prostaglandin synthesis and expression of inducible cyclooxygenase-2, an enzyme that mediates mechanical loading-induced bone formation *in vivo*. The response of osteocytes to fluid flow includes a rapid production of nitric oxide, and expression of endothelial nitric oxide synthase. Nitric oxide has been shown to mediate the mechanical effects in bone, leading to enhanced prostaglandin E_2 release.

Disruption of the actin-cytoskeleton abolishes the prostaglandin response to mechanical stress in osteocytes, suggesting that the cytoskeleton is involved in cellular mechanotransduction. These studies have increased our understanding of the cell biology underlying Wolff's Law. This may lead to new strategies for combating disuse-related osteoporosis, and may also be of use in understanding and predicting the long-term integration of bone-replacing implants.

Key words: *bone remodeling, osteocyte, mechanotransduction, fluid shear stress, nitric oxide*

1. Introduction

1.1. The Osteocyte Network in Bone

Osteocytes are stellate shaped cells enclosed within the lacuno-canalicular network of bone. The lacunae contain the cell bodies, from which the 5–30 μm long cytoplasmic processes (50–60 per osteocyte) radiate through the bone matrix via canaliculi (Fig. 1). The formation of these osteocyte processes is highly dependent on continuous cleavage of type-I collagen [1]. Osteocytes are connected by their cell processes to neighboring osteocytes and to the cells lining the bone surface. Some osteocyte processes pass through the lining cell layer thereby establishing contact with the extraosseus space [2], suggesting

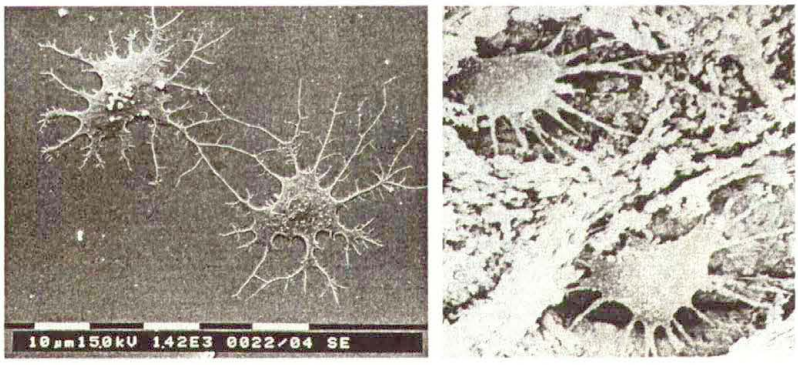

FIGURE 1. Osteocyte morphology. Left: Isolated osteocytes in culture. Osteocytes were isolated by an immunodissection method using MAb OB7.3-coated magnetic beads. After isolation the cells were seeded on a glass support, cultured for 24 hr and studied with a scanning electron microscope. After attachment, osteocytes form cytoplasmic extrusions in all directions. Right: Osteocytes embedded in calcified bone matrix. Note the many cell processes, radiating from the osteocyte cell bodies, as visualized using scanning electron microscopy. Magnification: 1000×. Micrograph kindly provided by Dr. P.J. Nijweide.

cell communication between the osteocyte and the osteoblast stem cell in the bone marrow compartments.

Dramatic changes occur in the distribution of actin-binding proteins during terminal differentiation of osteoblasts to osteocytes [3]. These changes likely contribute to the typical morphology of the osteocyte, which was originally thought to be enforced on differentiating osteoblasts during their incorporation in the bone matrix [4]. Osteocytes remain in contact with neighbouring osteocytes and with bone surface cells to ensure the access of oxygen and nutrients. Osteocytes in vitro express the typical morphology again as soon as they attach to a support [5] (Fig. 1). In bone, interactions between osteocytes-osteocytes and osteocytes-osteoblasts are achieved through cadherin-based adherens junctions and gap junctions [6]. Within each osteon therefore, osteocytes form a network of gap junction-coupled cells, representing an intracellular as well as an extracellular network system.

Which stimuli do "excite" the osteocytes? The answer likely comes from studies in which biomechanical concepts and techniques are applied to bone cell biology. Over the last decade, both theoretical and experimental data have led to the general notion that osteocytes are the pivotal cells in the biomechanical regulation of bone mass and structure [7–14]. The development of osteocyte isolation techniques, with the use of highly sensitive (immuno) cytochemical and *in situ* hybridization procedures, and molecular biological methods, are rapidly increasing our knowledge about the osteocyte, the least understood bone cell type. In this paper we will focus on the role of osteocytes in bone mechanotransduction, with emphasis on their role in determining the bone's structure.

1.2. Osteocyte Isolation from Bone

Osteocytes are terminally differentiated cells, embedded in hard matrix, and therefore have not received adequate attention, until a decade ago. The field has opened up by the group of Nijweide [5], who succeeded in the isolation and purification of chicken osteocytes from mixed bone cell populations obtained from fetal bones by enzymatic digestion, using an osteocyte-specific antibody directed to an antigen on the cytoplasmic membrane and an immunodissection method. The isolated osteocytes did behave similarly to osteocytes *in vivo*, i.e. the post-mitotic osteocytes re-acquired a stellate morphology and formed a cellular network by means of their long cell processes.

1.3. Osteocyte Markers

Characteristic for osteocytes is their location within the bone matrix, their typical stellate morphology and cytoskeletal organization, which is important for the osteocyte's response to loading [15]. The prominent actin bundles in the osteocytic processes in combination with the abundance of the actin-bundling protein fimbrin are typical for osteocytes [16]. Furthermore, osteocytes express osteocalcin, osteonectin, and osteopontin, but show little alkaline phosphatase activity [17].

Currently, the best markers for isolated osteocytes are their typical morphology [5] and their reaction with osteocyte-specific monoclonal antibodies [18, 19]. In addition, matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes [20], as well as the carboxyl-terminal PTH receptor (CPTHR), whose activation is involved in cell survival and communication [21], and sclerostin, an osteocyte-expressed negative regulator of bone formation [22]. Finally, dentin matrix protein 1 (DMP1) is also highly expressed in osteocytes and is mechanically responsive.

2. The Function of Osteocytes

2.1. Osteocytes as Mechanosensor Cells

Osteocytes are likely the mechanosensory cells *par excellence* of bone [9], dispersed throughout the mineralized matrix. Osteocyte density varies depending on skeletal site and developmental history [23]. They are directly connected with each other via gap junction-coupled [6] long slender cell processes running in canaliculi of unmineralized matrix. Superficial osteocytes are connected with lining cells and osteoblasts. An intracellular as well as an extracellular route allows for rapid passage of ions and signal molecules, enabling cellular signalling from osteocytes deep within the bone tissue to surface lining cells and vice versa.

Osteocytes are sensitive to applied stress [10, 24–29]. For example, intermittent loading at physiological strain magnitude produces rapid changes of metabolic activity in osteocytes [10]. Finite element simulation of bone remodeling, assuming this to be a self-organizational control process, also predicted a role for osteocytes as stress sensors of bone [11]. The mechanical environment of the stress-sensitive osteocyte varies with the geometry of the osteocyte lacuna [30].

How do osteocytes sense mechanical loading? Over the last few years a number of theoretical and experimental studies have appeared that agree that flow of interstitial fluid is likely the stress-derived factor that informs the bone cells about mechanical loading. In this view, the canaliculi are the bone porosity of interest, and the osteocytes the mechanosensor cells.

2.2. Interstitial Fluid Flow in Bone

There is early evidence of stress-induced fluid flow in bone [31], as well as of a substantial flow of fluid through the mineralized portion of bone [32]. The diameter of the canaliculi allows the stress-derived fluid flow to reach even the outermost osteocytes of an osteon [33]. Wang et al. [34] have delineated the bone's interstitial fluid pathway in vivo. These studies emphasized the importance of mechanically induced flow for the transport of metabolites to and from osteocytes in an osteon, to ensure osteocyte viability.

2.3. Effects of Fluid Flow on Osteocytes

Klein-Nulend et al. [10] subjected osteocytes, osteoblasts, and periosteal fibroblasts from chicken calvarial bone [5, 18] to two stress regimes, hydrostatic compression (IHC) and pulsatile fluid flow (PFF) [35]. Under both stress regimes, osteocytes appeared more sensitive than osteoblasts, and osteoblasts more sensitive than periosteal fibroblasts. In addition, osteocytes were particularly sensitive to fluid shear stress, more so than to hydrostatic stress. These conclusions are remarkably in agreement with the theory developed by the group of Cowin [9, 14, 36] that osteocytes are the professional mechanosensory cells of bone, and that they detect mechanical loading events by the canalicular flow of interstitial fluid that results from that loading event.

3. Mechanotransduction in Bone

Mechanotransduction is the process by which the mechanosensor cells convert the mechanical stimulus into intracellular signals. How the mechanical signal is detected and converted into a chemical, intracellular response has yet to be established. The composition and structure of the matrix in the periosteocytic sheath and the adherence of osteocytes to their surrounding matrix appear very important. The matrix composition and structure determines the bone's porosity for fluid flow and therefore the magnitude

of the fluid shear stress [14]. The osteocyte processes and their surrounding matrix possess a range of structural elements, which according to a theoretical model [37] should allow for a dramatic amplification of cellular-level strains [38]. As osteocytes are still capable of producing matrix proteins and proteoglycans, they might even modify their responsiveness to mechanical loading by adapting the matrix around them, and thereby the porosity of the lacuno-canalicular system.

Osteocytes adhere to their surrounding matrix by cytoplasmic membrane receptors such as integrins and CD44 receptors, coupled to the cytoskeleton. These receptors are likely the first step of intracellular signal transduction after mechanical stimulation [39]. Regulation of the number of adhesion sites and/or their coupling to intracellular signal transduction pathways might provide a mechanism by which endocrine modulation of the mechanoregulation of bone occurs. Extracellular Na^+ and Ca^{2+} influxes are believed to be necessary for downstream anabolic effects of mechanotransduction in osteocytes [40].

4. Stress Response and Cellular Deformation

The rate of the applied loading stimulus correlates to bone formation rather than the magnitude of strain [14, 41]. Low magnitude ($< 10\,\mu\varepsilon$), high frequency (10–100 Hz) loading can stimulate bone growth and inhibit disuse osteoporosis [42]. Also, high-magnitude, low-frequency stimuli are quite rare in the activities of daily life, whereas high-frequency, low-magnitude stimuli are quite common [42]. High rates of loading also increased bone mass and strength after jumping exercises in middle-aged osteopenic ovariectomized rats [43]. The rate of loading seems to be a decisive factor in bone formation and maintenance. However the underlying cellular mechanism is not well understood.

Bacabac et al. [35] found a linear correlation between increased amounts of nitric oxide (NO) released by bone cells and the rate of fluid shear stress (Fig. 2). Since NO mediates bone formation *in vivo* [44], this supports the notion that bone formation *in vivo* is stimulated by dynamic rather than static loads [45], and that low-magnitude, high-frequency mechanical stimuli may be as stimulatory as high-amplitude, low-frequency stimuli. This rate-dependent response was found to occur, provided that the cells are "kicked" in a pre-conditioned state [46]. The finding that the bone cell's response to

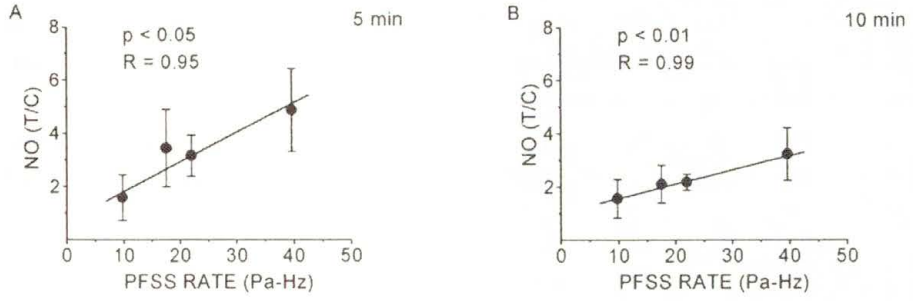

FIGURE 2. Nitric oxide production by bone cells is linearly proportional to the rate of fluid shear stress. The steepest slope was found at 5 min. (0.11 Pa-Hz^{-1}), indicating that the highest bone cell response to fluid shear stress rate occurs rapidly. At 10 min., NO levels were lower than those found at 5 min. Values are mean treatment-over-control ratios $(T/C)\pm$SEM.

fluid shear stress is rate-dependent provides an explanation why adaptive bone formation can occur despite the sporadic occurrence of high-amplitude strains in daily life [47].

The excitation mechanism of osteocytes might be due to a unique strain amplification that results from the interaction of the pericellular matrix and the cell process cytoskeleton [48]. This also provides an explanation for sustained bone formation despite the sporadic occurrence of high amplitude strains in normal physiological loading conditions. The theoretical approach leads to an extracellular mechanism for amplifying stress, whereas, experimental investigations leading to a rate-dependent response, provided a cellular basis for understanding the osteogenic adaptation of bone to mechanical loading. Further understanding of how bone cells respond to stress at the cellular level might provide a deeper insight on how bone copes with meager amounts of high amplitude loading.

Recent studies on the osteogenic activity of bone cells investigated the effects of stress using varying techniques (fluid flow, substrate strain, hydrostatic pressure, vibration stress [49]). The magnitude (and rate) of stress were shown to correlate with the cellular response, which was likely cell deformation-dependent. Relating the effects of fluid flow and substrate straining have shown that the former induces higher release of signaling molecules [50]. A numerical study confirmed that the cellular deformation caused by stress induced by fluid flow is fundamentally different from that induced by substrate straining [50]. Fluid shear stress has a larger overturning effect on the bone cells, while the effect of substrate strain is focused on cell-substrate

attachments. These recent results confirm the importance of investigating how cells deform in response to stress for understanding the corresponding physiological response of cells [50].

One possible pathway for the detection of strain involves mechanosensitive channels, since their sensitivity to membrane strain and tension may be relevant in the understanding of cellular reactions to mechanical strain and bone physiology [51].

5. Signal Transduction in Mechanosensing

To respond to mechanical stimuli with the production of signal molecules which modulate the activities of osteoblasts and osteoclasts, the mechanosensitive osteocytes have to convert the mechanical stimuli into intracellular signals (mechanotransduction). Extracellular matrix receptors, attached to the extracellular matrix as well as to the cytoskeleton, are prime candidates as mechanotransducers [39]. It is likely that the known intracellular signal transduction pathways, such as the intracellular Ca^{2+}, IP3, or cAMP dependent pathways, shown to play a role in other mechanosensitive cells, are involved [52]. Thereafter signal molecules are produced and secreted, such as prostaglandins and nitric oxide (NO) [53]. Osteopontin is rapidly upregulated by osteocytes after acute disuse, which may serve to mediate bone resorption, given that osteopontin acts as an osteoclast chemotaxant, and a modulator of osteoclastic attachment to bone [54].

Prostaglandins are involved in the response of bone tissue and cells to stress [55–59]. Both bone resorption caused by immobilization and bone formation caused by mechanical loading are inhibited by indomethacin in vivo. The early upregulation of prostaglandin release in response to mechanical stress was associated with induction of cyclooxygenase-2 (COX-2) [60], and an increase in the number of functional gap junctions [71]. Induction of COX-2 by stress might explain why prostaglandin production is continued for several hours after stress was stopped [25] and could be related to the memory phenomenon described *in vivo* [61].

NO produced by nitric oxide synthase (NOS) is an important mediator of the response of bone to stress. Several studies [10, 35, 42, 45, 60] have shown that NO production is rapidly increased in response to mechanical stress in bone cells, including isolated osteocytes (Fig. 2). In fracture patients, the proportions of osteocytes expressing NOS were reduced, suggesting that the

capacity to generate NO might be reduced as a result of fracture, leading to impairment of the ability of NO to minimize resorption [62]. The rapid release by mechanically stressed bone cells makes NO an interesting candidate for intercellular communication within the three-dimensional network of bone cells.

6. Single Cell Response to Mechanical Loading

Osteocytes' response to different kinds of mechanical loading has predominantly been studied in cell cultures or entire bone. However, single-cell level mechanosensing and chemical signaling in osteocytes is essential for bone adaptation [8]. The mechanosensitive part of the osteocytes, the cell body or the cell processes, has not been determined yet. Hence, information is needed on the single osteocyte's response to a localized mechanical stimulation. NO is an important marker for studying mechanosensitivity of osteocytes [25], and it mediates the induction of bone formation by mechanical loading *in vivo* [44]. NO is produced as a by-product when L-arginine is converted to L-citruline in the presence of nitrogen oxide synthase enzyme, molecular oxygen, NADPH and other cofactors [63]. It metabolises very fast and has a physiological half life of 5–15 seconds, making its direct online

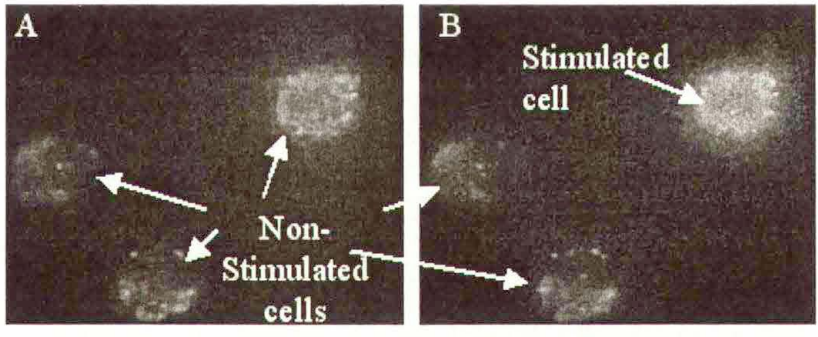

Figure 3. Fluorescence images showing intracellular NO production in MC3T3-E1 osteoblastic cells. (A) before mechanical stimulation (basal level NO production), and (B) after mechanical stimulation of a single cell. DAR 4M AM rhodamine loaded, surface-attached MC3T3-E1 cells were subjected to oscillatory mechanical loading (144–560 pN, 0.5–3 Hz, 1 min.) through cell-attached colloid particles using optical tweezers. The stimulated cell shows upregulation of intracellular NO production seen as increase in fluorescence intensity after mechanical stimulation.

detection difficult. DAR-4M AM is a recently developed NO indicator, which has been used to show upregulation of intracellular NO production in single osteocytes after localised mechanical stimulation [64] (Fig. 3). This unique technique, which allows real-time monitoring of chemical signaling at the single osteocyte level, might lead to a better understanding of the dynamic processes involved in mechanosensing.

7. Strain-dependent Regulation of Osteoclast Activity During Bone Remodeling

Throughout life, bone tissue renews itself by means of basic multicellular units (BMUs), consisting of osteoclasts and osteoblasts acting in a coordinated fashion to resorb existing bone and form new bone. The osteoclasts start to dig a tunnel through compact bone, and the osteoblasts refill the tunnel. In osteons there is always an open central canal of connective tissue with blood vessels and neurons. How do osteoclasts and osteoblasts collaborate to produce such osteons running along the direction of dominant strain of a bone piece?

It is unclear how the resorbing osteoclasts find their way through the pre-existing bone matrix during remodeling. The alignment of secondary osteons along the dominant loading direction suggests that remodeling is guided by mechanical strain, indicating that mechanical adaptation occurs throughout life at each remodeling cycle. Burger et al. [8] has proposed that alignment during remodeling occurs as a result of different canalicular flow patterns around cutting cone and reversal zone during loading. Low canalicular flow around the tip of the cutting cone reduces NO production by local osteocytes thereby causing their apoptosis (Fig. 4). Osteocyte apoptosis attracts osteoclasts, leading to further excavation of bone in the direction of loading. At the transition between cutting cone and reversal zone, enhanced canalicular flow stimulates osteocytes to release NO, which induces osteoclast retraction from the bone surface. Together a treadmill exists of attaching and detaching osteoclasts in the tip and the periphery of the cutting cone, and the digging of a tunnel in the direction of loading (Fig. 5).

How are the different strains around cutting and closing cone sensed by osteoclasts and osteoblasts? Mechanosensing in bone is primarily a task for the *osteocytes*, which likely translate the canalicular flow into osteoclast and osteoblast-recruiting cell signals. Therefore, if local strain differences around the cutting- and closing cone of a BMU regulate osteoclast and osteoblast ac-

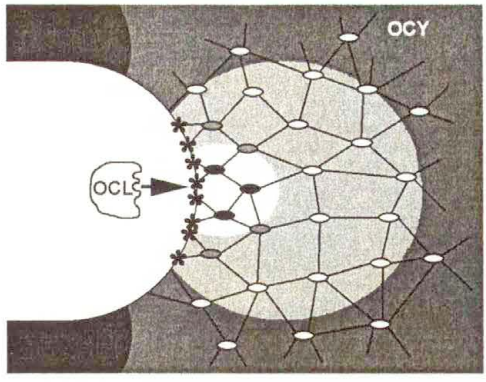

FIGURE 4. Cartoon of the cutting cone tip, showing the relation between apoptotic osteocytes and a progressing osteoclast. Osteocyte apoptosis (indicated as black lacunae) is caused by canalicular stasis, which directly results from the volumetric strain pattern caused by cyclic loading in the normal direction. As osteoclasts are attracted towards apoptotic osteocytes because of changes on the apoptotic cell surface (indicated by asterisks), the direction of the osteoclastic attack follows the direction of loading. OCY, osteocyte network; OCL, osteoclast.

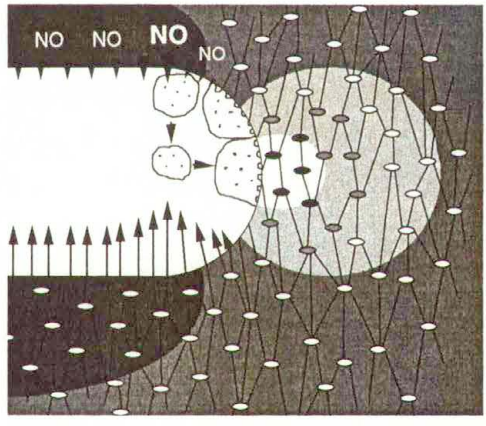

FIGURE 5. Cartoon of postulated events in the cutting cone of a progressing BMU. Osteoclasts are attracted by apoptotic osteocytes in the cutting cone tip, but forced to withdraw again from the bone surface at the cutting cone base, as a result of high amounts of NO produced by well stressed osteocytes. As NO production remains high further down the reversal zone, osteoclasts remain within the cutting cone and may even re-enter the resorption cycle, leading to a "treadmill" of active and inactive osteoclasts that together dig the resorption tunnel or trench. Vertical arrows indicate direction and magnitude of canalicular fluid flow; vertical arrow heads indicate release of NO by well stressed osteocytes.

tivity, these strain gradients must produce local canalicular flow differences that can be related to the recruitment of these two cell types. Volumetric strain in the bone around a BMU cutting cone was related to canalicular fluid flow [12, 13]. A mechanism was proposed explaining osteoclast behaviour in the cutting cone [8], based on the effect of different flow patterns on the osteocytes. Osteocytes produce high levels of nitric oxide (NO) in response to fluid shear stress [25]. Interestingly, endothelial cell nitric oxide synthase (ecNOS) is specifically involved in the cellular response to fluid shear stress [65]. NO production in response to adequate shear stress protects endothelial cells against apoptosis [66], and such a mechanism might also operate in bone, where osteocytes are protected against apoptosis by NO produced under normal canalicular shear stress. At the tip of the cutting cone osteocytes enter apoptosis as a result of insufficient NO production due to insufficient canalicular fluid flow [8], (Fig. 5).

Apoptotic osteocytes at the tip of the cutting cone will attract osteoclasts [67], that resorb the bone matrix and phagocytose the dying osteocytes, which may be the signal for osteoclasts to continue resorption in that direction. Osteocyte viability may thus play a significant role in the maintenance and integrity of bone [68]. Inhibition of osteocyte apoptosis is likely to affect bone remodeling. Most importantly, mechanical loading by hydrostatic pressure [69] and by fluid shear stress promotes osteocyte survival [88]. This is of interest in the context of stress-shielding-related osteocyte apoptosis, which could be the cause of continued tunneling by the osteoclasts of a BMU in the direction of loading, leading to stress-aligned osteonic remodeling [68].

The mechanism discussed above can explain why osteoclasts move in the direction of loading. At the base of the cutting cone and further down the reversal zone, osteocytes receive enhanced fluid shear stress during loading. NO production will therefore be even higher than normal, particularly by the superficial osteocytes, which will prevent futher osteocyte apoptosis, but may also promote the retraction and detachment of osteoclasts from the bone surface [70]. This may also occur at the base of the cutting cone. NO acts locally as a paracrine cell modulator, which perfectly suites a role as a very local inhibitor of further osteoclastic attack.

These two mechanisms, attraction of osteoclasts to the cutting cone tip and induction of osteoclast detachment from the cutting cone base, together explain the mechanically meaningful behaviour of osteoclasts during remodeling.

8. Conclusions

Important progress has been made over the last few years regarding the understanding of the role of osteocytes in bone metabolism and turnover. The collaboration between experimental investigation and theoretical analysis of the response of bone cells to stress has proven effective for advanced understanding of underlying processes in bone mechanotransduction. These studies agree that the network of osteocytes, perhaps in conjunction with the bone lining cells, provides the cellular structure that allows bone organs to determine local needs for bone augmentation or reduction in response to mechanical demands.

The role of osteocytes, as the mechanosensors in bone *par excellance*, has been elucidated in the past years with more detail. Computational models for cells have previously treated the cytoplasm and cytoskeleton as a continuum, but the importance of the cytoskeleton was recognized leading to more recent approaches which treat the cytoskeleton more closely to its physical reality, as consisting of interconnected fibers. Cell models combining many structurally significant cellular components may prove to be valuable tools in investigating the effect of varying stress applications at the cellular level.

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